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Genetic Dissection of the Role of Heparan Sulfate in Mammary Tumor Progression

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14. ABSTRACT
There is accumulating evidence that heparan sulfate (HS) controls various growth factor signaling events. There is also evidence that cellular HS production itself exerts strong influences on tumorigenesis, exemplified by the fact that mutations of *Ext1*, the gene encoding an HS synthesizing enzyme, cause multiple bone tumors. Furthermore, the level of HS degrading activity correlates with the aggressiveness of the tumor. Despite these long-standing observations, much less is known about the mechanisms by which HS influences the malignant behavior of tumors in vivo. Also important is the fact that HS is produced not only by tumor cells themselves but also by stromal cells that constitute the tumor microenvironment. This project will address these key issues by using genetic mouse models. The second year of this project was dedicated to conduct tumorigenesis studies that form the core of the project. Preliminary results suggest that HS indeed affects the progression of mammary tumors. We will continue these experiments during the third year to obtain statistically significant survival data. Analysis of tumors formed in these mice will shed light on the molecular mechanisms by which HS regulates mammary tumor progression.

15. SUBJECT TERMS
Heparan sulfate; Ext1; Tumorigenesis; Tumor microenvironment; Conditional knockout

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Table of Contents

Introduction

Heparan sulfate is a linear polysaccharide composed of repeating *N*-acetylglucosamine and glucuronic acid residues. The anticoagulant heparin is a specialized form of heparan sulfate. Heparan sulfate chains are covalently attached to various core proteins to form heparan sulfate proteoglycans (HSPGs). HSPGs exist mainly as cell surface and extracellular matrix molecules and are functionally involved in various biological processes, including growth factor signaling, regulation of morphogen gradient, cell adhesion, lipoprotein metabolism, and modulation of proteinase activities (Gallagher and Lyon, 2000). Considering its interactions with a number of growth factors/cytokines, it is likely that heparan sulfate plays an important role in cancer development and progression. For instance, two signaling molecules that have strong implications in human breast cancer, namely Wnt1 and neuregulin (the Ig-domain containing isoforms), bind and functionally modulated by heparan sulfate. Factors known to affect invasion, metastasis, and tumor angiogenesis, such as matrix metalloproteinases, VEGF, and endostatin, also interact with heparan sulfate. Despite this wealth of data, our understanding of the mechanisms by which heparan sulfate influences tumor cell behavior *in vivo* is still fragmentary. One of the important unknowns is what is the overall physiological effect of heparan sulfate on tumor development and progression. Further compounding the issue is that heparan sulfate is produced not only by tumor cells themselves but also by stromal cells within tumors. The field needs advanced animal models that not only closely mimic clinical cancers but also allow precise dissection of heparan sulfate function in different cell types. The key glycosyltransferase for the biosynthesis of heparan sulfate is the glycosyltransferase called EXT1. EXT1 catalyzes the polymerization of *N*-acetylglucosamine and glucuronic acid residues (Duncan et al., 2001; Zak et al, 2002). Genetic and biochemical studies have established that the *Ext1* gene is absolutely essential for heparan sulfate biosynthesis (Lin et al., 2000). These properties make *Ext1* as an excellent target for genetic disruption of heparan sulfate synthesis. This allows direct interpretation of the causal relationship between heparan sulfate and the resultant phenotype. Our primary objective is to obtain direct information regarding the role of heparan sulfate in breast cancer development and progression in the context of *de novo* mammary tumorigenesis models. An ancillary objective is to determine whether tumor cell-expressed heparan sulfate and stromal cell-expressed heparan sulfate exert distinct effects on the behavior of mammary tumors. We hypothesize that they have different effects on the growth and progression of mammary tumors. Through this project, we will define the role of heparan sulfate in breast cancer under the condition that mimics human breast cancer than ever before, thereby advancing our understanding of the role of heparan sulfate in breast cancer to the next level.

Body

Task 1. Acquisition of animal experiment approval.
Necessary approval for animal experiments was obtained on schedule.

Task. 2. Generation of animal cohorts for studies in Aim 1 (the role of tumor cell autonomous heparan sulfate in mammary tumor development and progression).
As reported in the previous progress report, this part of the project had experienced a problem of low fertility of *KFS2MT6* transgenic mice (Cecena et al., 2006) during the first year, which incurred a slight delay in this part of the project. This problem has now been corrected by obtaining new breeding pairs from Dr. Robert Oshima, the creator of the transgenic line. Despite this delay, tumorigenesis study has been initiated, and, thus far, we have obtained a preliminary tumor free curve for heterozygous conditional knockout mice (Fig. 1). During the third year of this project, we expect to complete this study by analyzing sufficient numbers of homozygous and heterozygous conditional *Ext1* knockout mice.

Task 3. Analysis of mammary tumors in Aim 1.
As stated above, the tumorigenesis study on the *KFS2MT6;MMTV-Cre;Ext1*^{flox} model had experienced a delay during the first year. As a result, the mice are still under the tumorigenesis study and tumor tissues cannot be obtained until the predefined endpoint of the study. We expect to initiate this task during the first four months of the third year. Meanwhile, we have established the analytical methods for mammary tumors, which will be employed for this study (Fig. 2). First tissue samples from *KFS2MT6;MMTV-Cre;Ext1*$^{flox/flox}$ and *KFS2MT6;MMTV-Cre;Ext1*$^{flox/wt}$ mice are expected to be examined sometime during the first two months of the third year.

Task 4. Expression profiling of genes between *Ext1* null and control tumors.
By the same reason as stated for Task 3, *KFS2MT6;MMTV-Cre;Ext1*$^{flox/flox}$ mice, *KFS2MT6;MMTV-Cre;Ext1*$^{flox/wt}$ mice, and their controls are still under the tumorigenesis study, and tumor tissues cannot be obtained until the predefined endpoint of the study. Meanwhile, we have accumulated enough expertise in expression profiling analysis through a variety of projects that have been conducted in the laboratory. Therefore, once tumor tissues become available, expression profiling experiments can be done quickly.

Task 5. Generation of animal cohorts for studies in Aim 2 (the role of stromally produced heparan sulfate in mammary tumor development and progression).
Because we have accelerated this part of the project to compensate the delay in Aim 1, we have made a greater progress in Aim 2 than in Aim 1 during the second year as summarized below.

FSP1-Cre;Ext1$^{flox/flox}$ mice develop normally. A concern with this Aim was that ablation of *Ext1* in FSP1-expressing fibroblasts may cause substantial developmental defects or even embryonic or neonatal death, which would essentially prevent us to perform the tumorigenesis studies as proposed. Therefore, we examined the development of *FSP1-Cre;Ext1*$^{flox/flox}$ mice. We found that these mutant mice grow normally without any gross abnormalities and are reproductive (not shown). Thus the proposed study can be done as planned.

Analysis of mammary development in *FSP1-Cre;Ext1*$^{flox/flox}$ mice. It is possible that conditional *Ext1* knockout in FSP1-expressing fibroblasts alter the normal development of the mammary glands, which would in turn complicate the analysis of HS function in mammary tumorigenesis. To rule out this possibility, we examined the mammary glands in *FSP1-Cre;Ext1*$^{flox/flox}$ mice and their control littermates. As shown in Fig. 3, mammary gland development in *FSP1-Cre;Ext1*$^{flox/flox}$ mice is indistinguishable from that in wild-type mice. This confirms that the proposed tumorigenesis study using *PyMT;FSP1-Cre;Ext1*$^{flox/flox}$ mice can be performed as planned.

Analysis of tumorigenesis in *PyMT;FSP1-Cre;Ext1*$^{flox/flox}$ mice. This is the main task in Aim 2. We have initiated this study during the second year and have obtained preliminary tumorigenesis data. Development of mammary tumors in *PyMT;FSP1-Cre;Ext1*$^{flox/flox}$ mice has been analyzed as tumor free curves (Fig. 4). The presence of tumors was examined by palpation as described in the original application. As shown in Fig. 4, mammary tumorigenesis in the *Ext1*$^{+/-}$ background in stromal fibroblasts appears to be accelerated. By 6 weeks, all *PyMT;FSP1-Cre;Ext1*$^{flox/wt}$ mice developed palpable tumors, which is substantially sooner than control mice. Thus far, 16 wild type and 4 *PyMT;FSP1-Cre;Ext1*$^{flox/wt}$ mutant mice have been included in this study. This preliminary result suggests that endogenous HS produced by stromal fibroblasts may exert an inhibitory effect on mammary tumorigenesis. Additional mutant mice, both the *PyMT;FSP1-Cre;Ext1*$^{flox/flox}$ and *PyMT;FSP1-Cre;Ext1*$^{flox/wt}$ genotypes, are currently being added to complete this study. In the third year, we will increase the number of animals to generate statistical data.

Task 6. Analysis of mammary tumors in Aim 2.

Analysis of lung metastasis. In the third year, we will examine lung metastasis in both *KFS2MT6;MMTV-Cre;Ext1*^{flox} and *PyMT;FSP1-Cre;Ext1*^{flox} models. As a preliminary study, we have examined lung metastasis in *PyMT;Ext1*$^{flox/flox}$ mice. This analysis was performed under the following criteria (Wang et al., 2008): (i) mice are at least 105 days old; (ii) palpable mammary tumors have been detected for at least 30 days; and (iii) the size of tumors is at least 1.5 cm in diameter. Fig. 5 shows the macroscopic and histological analyses of lung metastasis in a *PyMT;Ext1*$^{flox/flox}$ mouse. As anticipated, control mice under these criteria developed multiple foci of lung metastasis with a penetrance of 80%. Thus the study can be performed with *Ext1* mutant mice as planned. This study

will be performed on the animals that completed the tumorigenesis study. It is expected that we will be able to complete this study by the middle of the third year.

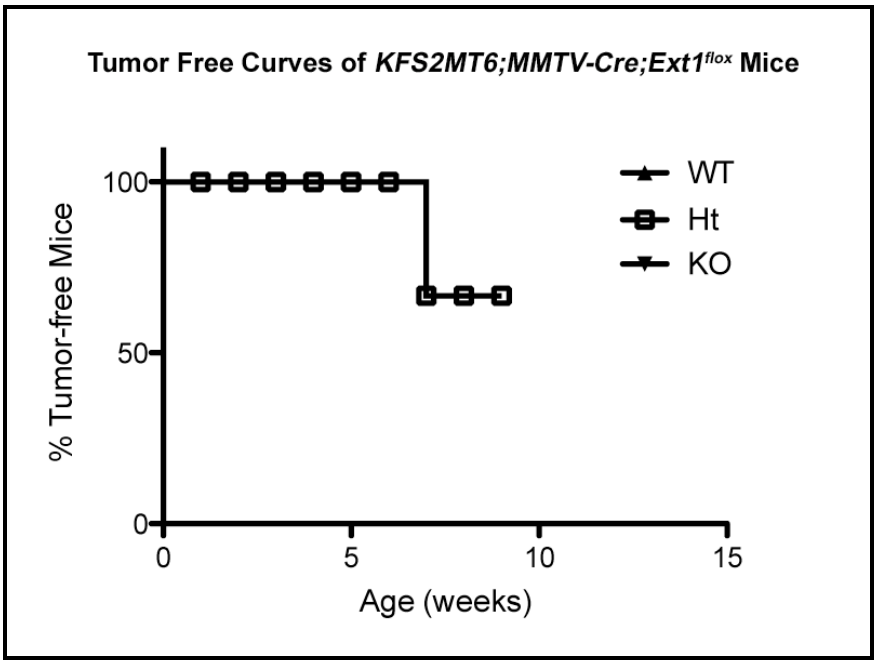

Figure 1. Preliminary results from the tumorigenesis study with the *KFS2MT6;MMTV-Cre;Ext1*^{flox} model. Thus far, three *KFS2MT6;MMTV-Cre;Ext1*$^{flox/wt}$ animals have been monitored up to 10 weeks. Currently, additional animals of different genotypes are being included in this study.

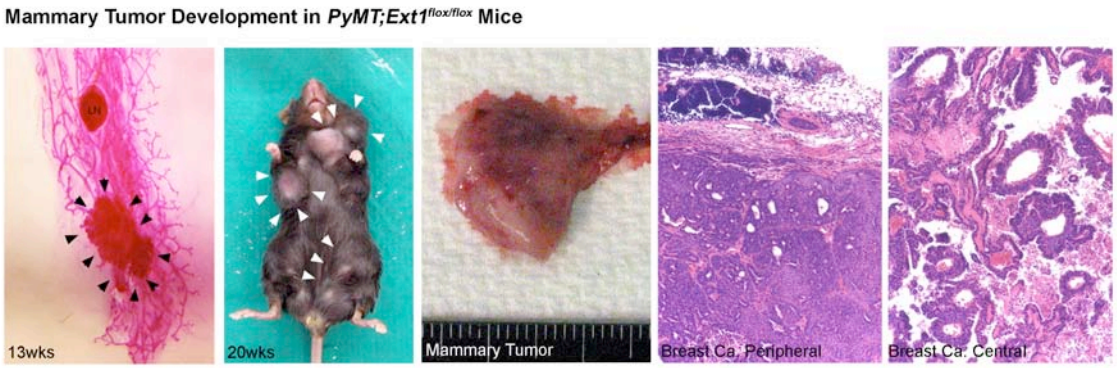

Figure 2. Characterization of mammary tumors developed in *PyMT;Ext1*$^{flox/flox}$ mice.

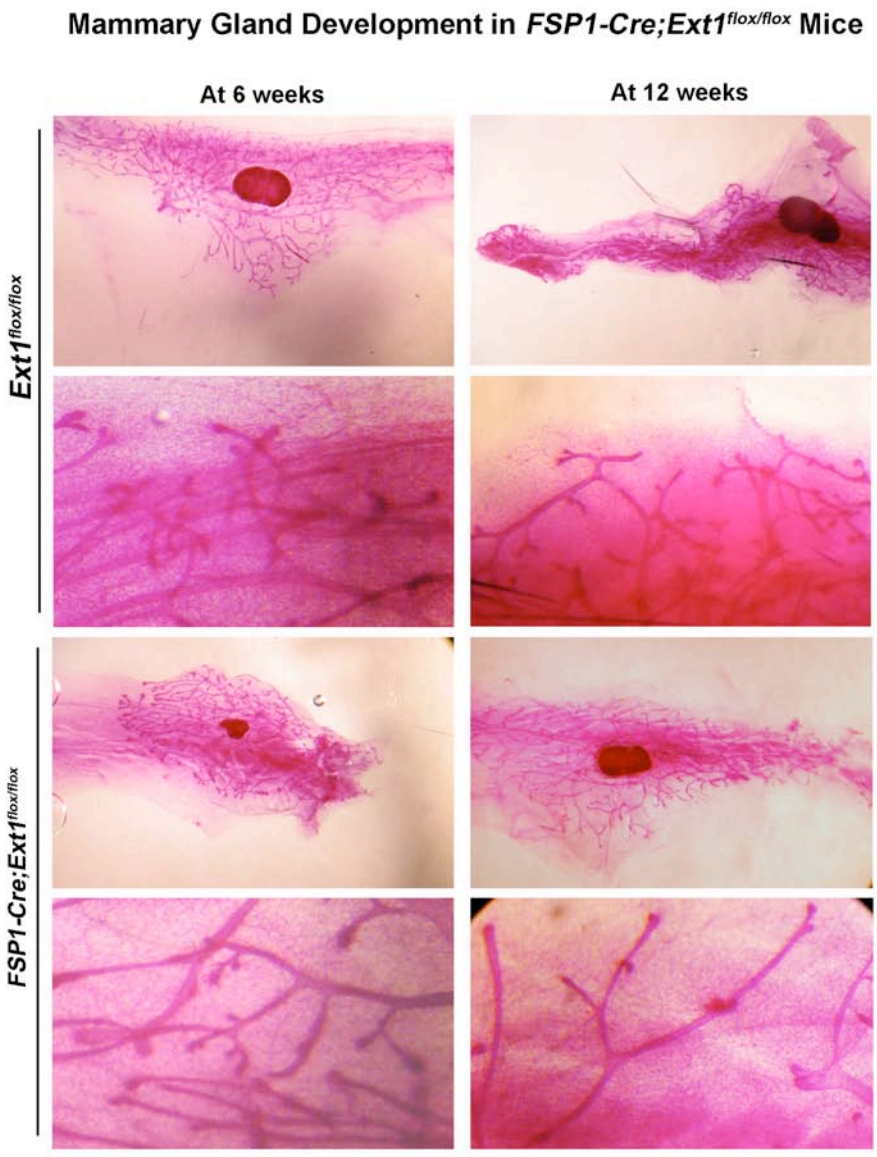

Figure 3. Mammary gland development in *FSP1-Cre;Ext1*$^{flox/flox}$ mice. Ablation of heparan sulfate in stromal fibroblasts does not significantly alter the pattern of mammary gland development.

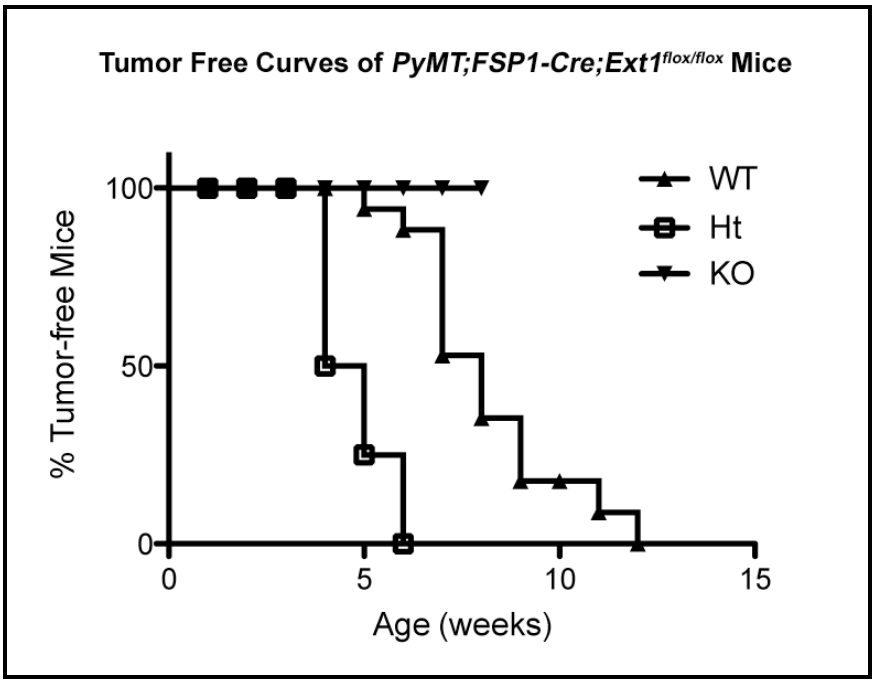

Figure 4. Preliminary results from the tumorigenesis study with the *PyMT;FSP1-Cre;Ext1*^{flox} model. Thus far, 16 wild type, 4 *PyMT;FSP1-Cre;Ext1*$^{flox/wt}$ heterozygotes, and one *PyMT;FSP1-Cre;Ext1*$^{flox/flox}$ homozygoutes have been monitored up to 12 weeks. Currently, additional heterozygous and homozygous animals are being included in this study.

Figure 5. Examination of lung metastasis in *PyMT;Ext1*$^{flox/flox}$ mice. Gross appearance of the animal and its lungs and H-E stained sections of the lung are shown.

Key Research Accomplishments

Breeding of experimental animals

- Produced experimental and control animals for tumorigenesis study in Aim 1 (the *KFS2MT6;MMTV-Cre;Ext1^{flox}* model). Breeding efforts will continue to produce a sufficient number of animals.
- Produced experimental and control animals for tumorigenesis study in Aim 2 (*PyMT;FSP1-Cre;Ext1^{flox}* model). Breeding efforts will continue to produce a sufficient number of animals.

Tumorigenesis study: KFS2MT6;MMTV-Cre;Ext1^{flox} model

- Produced experimental and control animals for tumorigenesis study. Preliminary results on tumorigenesis in *KFS2MT6;MMTV-Cre;Ext1$^{flox/wt}$* mice have been obtained.

Tumorigenesis study: PyMT;FSP1-Cre;Ext1^{flox} model

- Produced experimental and control animals for tumorigenesis study in Aim 2. Preliminary results on tumorigenesis in *PyMT;FSP1-Cre;Ext1$^{flox/wt}$* and control mice have been obtained.

Characterization of mammary gland development in FSP1-Cre;Ext1$^{flox/flox}$ mice

- Confirmed that mammary glands develop normally in *FSP1-Cre;Ext1$^{flox/flox}$* mice.

Characterization of mammary tumor development and lung metastasis in PyMT;Ext1$^{flox/flox}$ mice

- Confirmed that in control wild type mice, mammary tumors develop and metastasize according to the patterns previously reported for the PyMT model.

Development of analytic methods

- Established methods for histological characterization of normal mammary glands and mammary tumors.

Reportable Outcomes

- Abstract for DOD Era of Hope 2008 Meeting

  Matsumoto, K., and Yamaguchi, Y. "Genetic dissection of the role of heparan sulfate in mammary tumor progression"

Conclusion

This is the second progress report of this IDEA award. The goal of the project is to obtain physiological evidence for the role of heparan sulfate (HS) in mammary tumor development and progression using conditional knockout mouse models. Because of the complexity of the breeding schemes necessary for producing triple compound mutant mice and their control counterparts, the large portion of the grant period is to be spent for crossbreeding of mice. As reported last year, we have encountered breeding problems with *KFS2MT6* transgenic mice, which incurred some delay in Aim 1.

While the first year has been spent mostly for setting up colonies of necessary mouse models and initial steps of crossbreeding to produce various compound mutant mice, we could initiate during the second year mammary tumorigenesis studies which represents the core of this project. While tumorigenesis study in Aim 1 has slightly lagged behind the schedule due to the aforementioned breeding problem, we could still initiate this study and obtained preliminary data on *Ext1*$^{+/-}$ background. As for the tumorigenesis study in Aim 2, we are already in the middle of the experiment, which is expected to be completed during the first four months of the third year.

Overall, we have solved problems encountered during the first year and ruled out potential concerns that may have forced us to take an alternative *ex vivo* approach. We have also established the methods to characterize mammary tumors themselves and their lung metastatic foci. The *de novo* mammary tumorigenesis studies using *KFS2MT6;MMTV-Cre;Ext1*^{flox} and *PyMT;FSP1-Cre;Ext1*^{flox} models have been initiated during the second year. Thus, despite a slight delay, we will be able to complete these studies, as well as the characterization of tumor tissues, by the end of the third year.

Our preliminary data from the *PyMT;FSP1-Cre;Ext1*^{flox} model suggest that endogenous HS derived from stromal fibroblasts may exert an inhibitory effect on mammary tumorigenesis. If this preliminary observation is confirmed, it would provide novel insight into the possible use of heparin analogues as agents to inhibit the growth of mammary tumors.

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View Paper

GENETIC DISSECTION OF THE ROLE OF HEPARAN SULFATE IN MAMMARY TUMOR PROGRESSION

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Background and Objectives: Heparan sulfate (HS) exists mainly as cell surface and extracellular matrix molecules and are functionally involved in various biological processes. Considering its interactions with a number of growth factors/cytokines, it is likely that HS plays an important role in cancer development and progression. Two signaling molecules that have strong implications in human breast cancer, namely Wnt1 and neuregulin (the Ig-domain containing isoforms), bind and functionally modulated by HS. Factors known to affect invasion, metastasis, and tumor angiogenesis, such as matrix metalloproteinases, VEGF, and endostatin, also interact with HS. Despite this wealth of data, our understanding of the mechanisms by which HS influences tumor cell behavior in vivo is still fragmentary. One of the important unknownswhat is the overall physiological effect of HS on tumor development and progression. Further compounding the issue is that HS is produced not only by tumor cells themselves but also by stromal cells within tumors. The field needs advanced animal models that not only closely mimic clinical cancers but also allow precise dissection of HS function in different cell types.

Methodologies: Our experimental tool is the conditional Ext1 allele. The key glycosyltransferase for the HS biosynthetic process is the GlcNAc/GlcA copolymerase encoded by the Ext1 gene. Genetic and biochemical studies have established that EXT1 is absolutely essential for HS biosynthesis. We have created loxP-modified Ext1 allele, from which conditional Ext1 knockout mice can be generated by crossing with Cre transgenic mice targeted to various tissue and cell types. In this project, conditional ablation of the Ext1 gene will be combined with polyoma middle T antigen (PyMT)-dependent de novo mammary tumorigenesis models. We will produce MMTV-Cre;KFS2MT6;Ext1$^{flox/flox}$ animals (KFS2MT6 is a PyMT transgene that is activated by Cre-mediated excision of the STOP cassette). MMTV-Cre will activate PyMT expression and disrupt Ext1 concurrently in the mammary epithelium. Therefore, this system will allow us to examine specifically the role of tumor cell autonomous HS. We will also examine PyMT-induced mammary tumorigenesis in the HS-deficient stromal environment. For this, Ext1 will be disrupted specifically in stromal fibroblasts, without interfering HS synthesis in tumor cells, using the FSP1-Cre transgene. This experiment will allow us to determine the role of stromal cell-derived HS in tumor progression. In both experiments, mammary tumor progression will be analyzed in terms of growth, angiogenesis, invasion, and metastasis. Microarray analysis will be performed on tumor RNAs to gain insight into the difference in intracellular signaling between Ext1 null and control backgrounds.

Results to Date: We have completed backcrossing all necessary lines to C57BL/6 and are currently breeding experimental and control groups for the tumor analyses.

Conclusions: Considering its clear implication in tumor growth and progression, HS is an emerging therapeutic target in breast cancer. Yet our current concept on the role of HS in tumor development is derived mostly from the observations obtained from non-physiological experimental models. This project would have a direct impact on this issue by defining the physiological role of HS in mammary tumor development and progression.

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